Pectolytic Activity of Rice Plants Infected with the Stem-rot Fungi, *Helminthosporium sigmoideum* Cav. and *H. sigmoideum* var. *irregulare* Cr. et Tullis

by

Hei-ti Hsu[1]

Abstract

Production of pectolytic enzymes in stem tissues of both healthy and stem-rot infected plants were studied. The cut-stem technique was employed for production of pectolytic enzymes. Viscometric method and reducing end group analysis were used to determine endo-polygalacturonase and exo-polygalacturonase, while continuous titration procedure was used to measure the pectin mythylesterase activity.

Endo- and exo-polygalacturonase could nct be detected in tissue extracts from healthy rice stems. Extracts from infected tissues contained polygalacturonase activity. Pectin methylesterase activity was detected in healthy and diseased tissues. A considerable increase in pectin methylesterase activity of tissue extracts from infected stems was observed.

Introduction

Previous report (Hsu, 1967 a) demonstrated the pectolytic activities of exc-cellular enzymes in culture filtrates of the rice stem rct fungi, *Helminthosporium sigmoideum* and *H. sigmoideum* var. *irregulare.* Bcth endo-polygalacturcnase and exo-polygalactu-ronase were inducibly produced. Producticn of the enzymes in culture filtrates was influenced by the age of cultures. These studies provided evidence as to the capacity of the above pathogens to produce pectolytic enzymes. However, a further study is still needed in determining the role of pectolytic enzymes *in vivo.*

The present studies were made to ccmpare the production of pectolytic enzymes in infected and healthy tissues of rice plants. A preliminary repcrt has been published (Hsu, 1967 b).

Materials and Methods

Rice variety Chianung 242 was used as hcst plants. Same isclates cf these two fungi (Hsu, 1967 a), *H. sigmoideum* and *H. sigmoideum* var. *irregulare* used in previous

---

1.Former Research Assistant, Department of Plant Pathology, Taiwan Agricultural Research Institute, Taipei, Taiwan, China, now graduate assistant, Plant Virus Laboratory, Department of Botany, University of Illinois, Urbana, Illinois, U.S.A. Appreciation is expressed to Dr. S. H. Ou of the International Rice Research Institute, Los Baños, Laguna, Philippines and Dr. L.C. Wu of National Taiwan University, Taipei, Taiwan, China, for their encouragement and suggestion during the proceeding of the work.

study were employed and maintained on potato dextrose agar (PDA). The cut-stem technique described by Hsieh and Ou (1966) was modified to demonstrate the production of pectolytic activity in the infected tissues. The middle portions of the internode were disinfected with 70 percent ethyl alcohol and punctured with a bunch of needles to make wounds. The wounded portion was inoculated by wrapping a 5×10mm PDA bit on which the fungus had grown. The inoculated stems were placed upside down in a test tube. The test tubes with open ends downwards were supported on a rack which was placed in a tray with water just deep enough to keep the stem alive. They were kept at about 24°C and provided with natural light at day time. The water in the tray was changed every day to minimize contamination of microorganisms. Samples were taken 10 days after inoculation for polygalacturonase assay. For the pectin methylesterase assays, samples were taken every two days.

For the assay of enzymes only infected tissues were collected and carefully wiped with a piece of cotton to remove the agar bit and external mycelia from the stem. Five-gram samples were ground in a motar with 10 ml of distilled water for polygalacturonase preparation. In the case of pectin methylesterase, 0.25 M NaCl was added instead of using distilled water. The ground tissue was strained through 4 layer chessecloth and centrifuged at 8,000×g for 20 min. The supernatant adjusted to the desired pH constituted the enzyme preparation. Enzyme preparation was assayed immediately. Boiled enzyme preparation was used as controls.

Viscosity measurements and reducing group analysis were employed for the determination of polygalacturonase activity (Hsu, 1967 a). Ostwald-Fenske No. 200 viscosity pipetts were supported in a constant-temperature water bath at 30°C. Viscometric measurements were made in the tube containing 5 ml of 1 percent (W/V) pectin (Wako Pure Chemical Industries, Ltd.) or Na-polypectate (Cika Kanto Chemical Co., Ltd.) and 1 ml enzyme preparations buffered at desired pH with 0.05M citrate and 0.05M phosphate. Enzyme activities were expressed as the reciprocal of time (miuntes) for 50 percent loss in viscosity and multiplied with 100. Reducing group analysis for measurement of exo-polygalacturonase activity was performed by using the dinitrosalicylic acid (DNS) procedure (Miller, 1959) as modified by Hancock and Millar (1965). After 2 hr incubation period, 2.8 ml samples were removed from reaction mixtures and added to 0.2 ml 1 M Na_2CO_3 in test tubes. Three ml of the DNS reagent were then added to each tube. The tubes were held in a boiling water bath for 10 min to permit color reaction. The mixtures then were cooled immediately to room temperature and measured for color intensity at 575 mμ against a reagent blank containing boiled enzyme preparation with a Beckman spectrophotometer Model DU. The net increase of reducing materials in reaction mixtures was determined by comparing the optical density obtained from a standard curve prepared with galacturonic acid. Enzyme activities were expressed as μeq reducing groups per ml enzyme preparation after 2 hr incubation.

Pectin methylesterase activity was assayed by the method described by Kertesz (1955) with a slight modification. Fifty ml of 1.0 percent pectin solution containing 0.1 M NaCl was adjusted to pH 7.0 and placed in a water bath maintained at 30°C. Five ml enzyme preparation was then added and the time was noted. The pH was constantly maintained within 0.5 units of pH 7.0 for 30 min. The extent of demethylation resulting from pectin methylesterase activity was determined by titration with 0.02 N NaOH and expressed as μeq of methoxyl groups released in 30 min per ml enzyme preparation under the specified conditions.

Results

Endo- and exo-polygalacturonase could not be detected in extracts from healthy rice stems. Extracts of infected tissues contained polygalacturonase activity. Polygalacturonase (s) could be detected viscometrically from stem tissues infected by both ***Helminthosporium*** spp. (Fig. 1). The extracts of infected tissues upon incubation with pectin or Na-polypectate showed a maximum polygalacturonase activity at pH 4—5. These results were confirmed further by reducing group analysis (Fig 2). Reduction of substrate viscosity and increase in reducing power were observed in the extracts of infected tissues incubated with Na-polypectate at pH values 3—8. However, when pectin was used as substrate, the enzyme activity was relatively low and negligible at pH 7 and 8.

Pectin methylesterase activity was detectable in both healthy and diseased tissue by the continuous titration procedure. A considerable increase in pectin methylesterase activity of tissue extracted with 0.25 M NaCl from rice stems either infected with ***H. sigmoideum*** or ***H. sigmoideum*** var. ***irregulare*** is shown in Fig. 3. The pectin methylesterase activity in infected stem tissues was about 5 folds of that in healthy ones. An increase of pectin methylesterase activity in infected tissues was evident starting from 4 days after inoculation.

Discussion

Extracts of the ***Helminthosporium*** infected tissues contained pectolytic enzyme(s) degrading pectic substances. A maximum activity was obtained at pH 4 and 5 when either pectin or Na-polypectate was used as substrates and determination made by the viscometric method and reducing end group analysis. ***H. sigmoideum*** and ***H. sigmoideum*** var. ***irregulare*** produced endo- and exo-polygalacturonase in culture filtrates in the presence of pectic substances (Hsu, 1967 a). However, the present study did not clearly reveal that hydrolysis of pectic substances involved two enzyme systems. The pattern in which the pectic substances were hydrolyzed by the infected tissue extracts is difficult to explain. However, response of exo-polygalacturonase activity to the acidity of the reaction mixture was almost identical to that of enzyme produced ***in vitro***.

The presence of one enzyme in a crude preparation could be masked by the activity of the other enzyme. Tests of enzyme preparations on more than one substrate at different pH values proved this viewpoint. *In vitro* and *in vivo* studies emphasize the importance of enzyme sources and test conditions in determining apparent activity of particular pectolytic enzymes.

The exo-polygalacturonase associated with infected tissues is presumably of fungal origin, since neither could be demonstrated from healthy plant extracts. Kertesz (1951) also reported that polygalacturonase was not found as a normal constituent of higher plant tissues.

Although stem-rot fungi produced endo-polygalacturonase *in vitro*, its presence in infected tissue was not detected. Seemingly, endo-polygalacturonase does not appear to be significant in pathogenesis by *H. sigmoideum* and *H. sigmoideum* var. *irregulare*. Nevetheless, this enzyme may be important in saprogenesis, since it was produced in large quantities when the fungi were cultured on autoclaved rice stems or in liquid media containing pectic substances (Hsu, 1967 a).

Rice stems from both infected and healthy stems showed considerable pectin methylesterase activity. Extract from diseased tissues contained about 5 folds the pectin methylesterase activity of non-infected stems. However, pectin methylesterase could not be demonstrated for *H. sigmoideum* and *H. sigmoideum* var. *irregulare in vitro*. This suggested that the increased pectin methylesterase activity in diseased tissues was resulted from host-parasite interaction as previously suggested (Hsu, 1967 a). Pectin methylesterase in healthy plants was found to be bound to the cell walls (Jansen et al., 1960). Therefore, it might be implied that the infected stems become softened and macerated by polygalacturonase(s) of stem-rot fungi which, in turn, release the host pectin methylesterase.

Mode of infection by stem-rot fungi (Hsieh and Ou, 1966) and occurrence of exo-polygalacturonase and pectin methylesterase in infected tissues of rice stems are important for establishment and development of this disease. Further studies on production of pectolytic enzymes *in vivo* are needed for a better understanding of the roles of these enzymes in pathogenesis.

Literature Cited

Hancock, J. G., and R. J. Millar. 1965. Relative importance of polygalacturonate trans-eliminase and other pectolytic enzymes in southern anthracnose, spring black stem, and stemphylium leaf spot of alfalfa. Phytopathology 55:346--355.

Hsieh, S. P. Y., and S. H. Ou, 1966. The mode of infection by stem rot fungi. Abstracts of papers accepted for presentation at the third annual meeting of the Philippine Phytopathological Society.

Hsu, H. T. 1967a. Pectolytic activities of exocellular enzymes of the rice stem rot fungi. Plant Protection Bull. 9:15—22.

Hsu, H. T. 1967b. Degradation of pectic substances by the extract of stem-rot infected tissues of rice plants. (Abs. in Chinese). Plant Protection Bull. 9:97.

Jansen, E. F., R. Jang, and J. Bonner, 1960, Binding of enzymes to Avena coleoptile cell walls. Plant Physiol. 35:567-574.

Kertesz, Z. I. 1951. The pectic substances. 628 p. Interscience Publishers. New York.

Kertesz, Z. I. 1955. Pectic enzymes. p. 158-166. In S. P. Colowick and N. O. Kaplan (ed.) Methods in Enzymology. Vol. 1. Acad, Press. New York.

Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31:426-428.

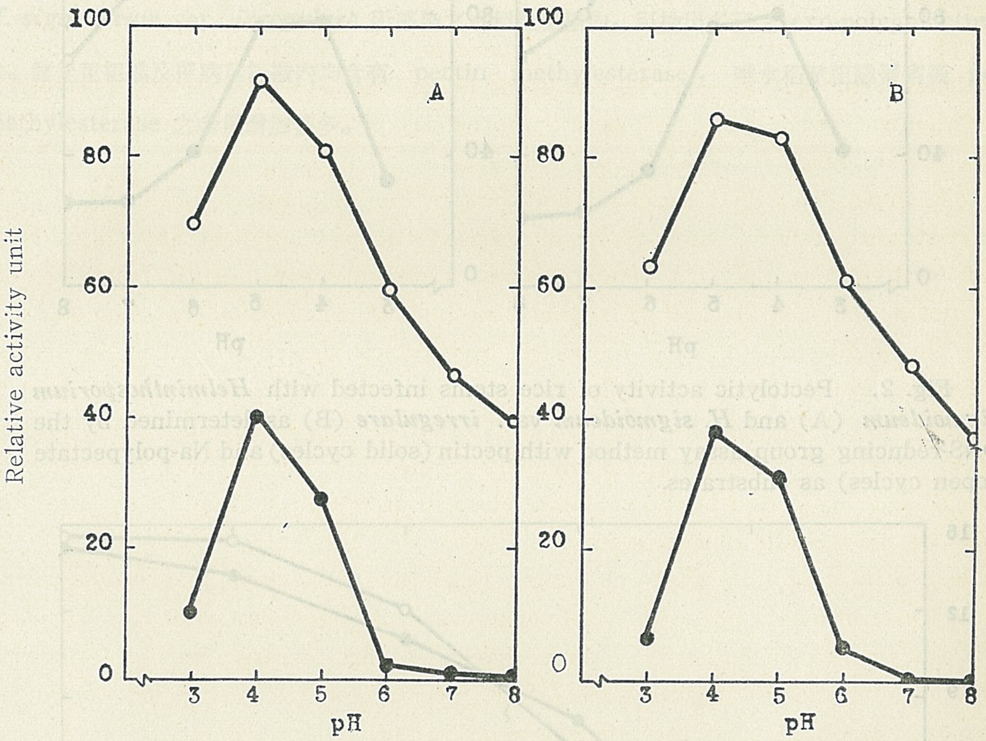

Fig. 1. Pectolytic activity of rice stems infected with *Helminthosporium sigmoideum* (A) and *H. sigmoideum* var. *irregulare* (B) as measured by viscometric procedure with pectin (solid cycles) and Na-polypectate (open cycles) as substrates.

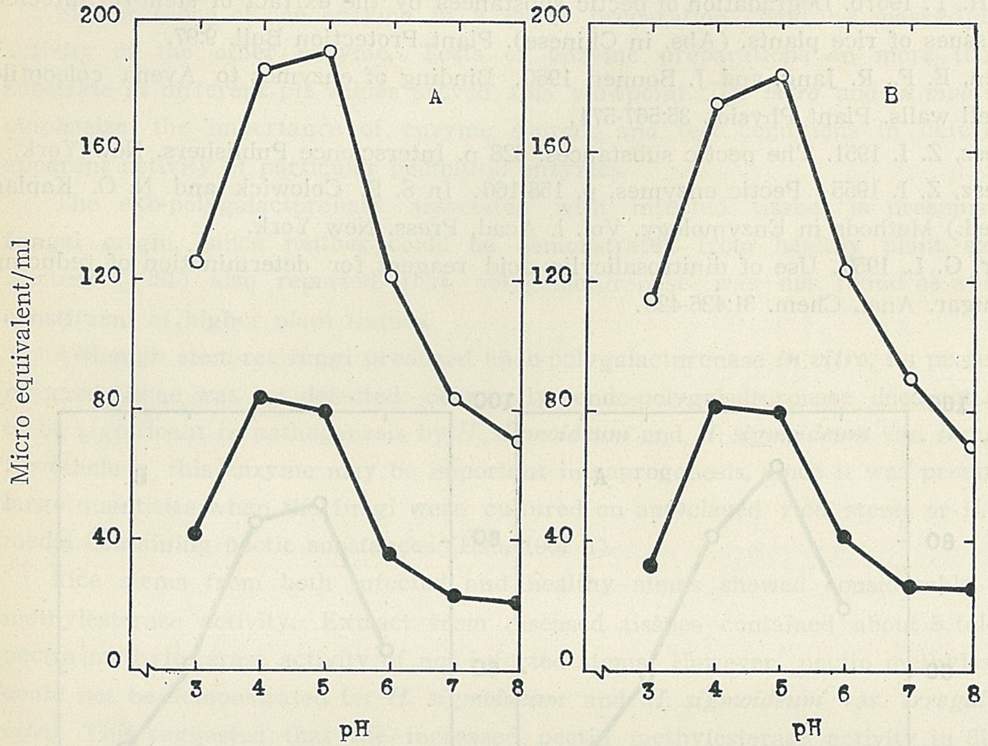

Fig. 2.　Pectolytic activity of rice stems infected with ***Helminthosporium sigmoideum*** (A) and ***H. sigmoideum*** var. ***irregulare*** (B) as determined by the DNS-reducing group assay method with pectin (solid cycles) and Na-polypectate (open cycles) as substrates.

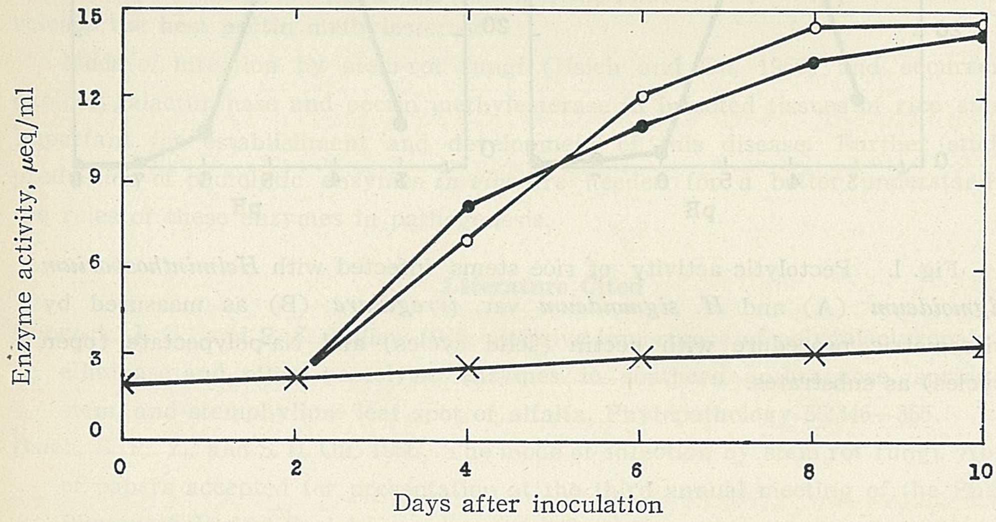

Fig. 3.　Pectin methylesterase activity of healthy (crosses), ***Helminthosporium sigmoideum*** infected (open cycles) and ***H. sigmoideum*** var. ***irregulare*** infected (solid cycles) tissues of rice stems as affected by the development of lesions.

水稻小球和小黑菌核病病原菌對寄主果膠分解酵素之影響

徐 惠 迪

本文敍述小球菌核病病原菌，***Helminthosporium sigmoideum*** Cav. 及小黑菌核病病原菌 ***H. sigmoideum*** var. ***irregulare*** Cr. et Tullis 對水稻體內果膠分解酵素產生之影響。健全水稻莖組織內沒有 endo-polygalacturonase 及 exo-polygalacturonase 之存在，被 ***H. sigmoideum*** 或 ***H. sigmoideum*** var. ***irregulare*** 所感染之水稻莖組織內，可檢出多量之 exo-polygalacturonase。健全莖組織及罹病莖組織內均含有 pectin methylesterase，唯水稻莖組織罹病後 pectin methylesterase 之含量增加甚多。